THE ROLE OF MAP3K1 IN LUMINAL SUBTYPE BREAST CANCER

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ABSTRACT

Mitogen-activated protein kinase kinase kinase 1, or MAP3K1, has been shown in a number of studies to be associated with luminal A subtypes of breast cancer. However, in the absence of any functional studies, whether this association is more than causative remains to be seen. I have knocked out MAP3K1 expression in both the non-tumorigenic MCF-10A cell line as well as the tumorigenic MCF-7 cell line in order to ascertain its role in breast cancer biology and development. Further, a number of other genetic alterations were carried out, including estrogen receptor expression, PIK3CA activation, and MAP2K4 silencing. MAP3K1 loss doesn't seem to have any effect on growth, morphology, or sensitivity to selected drugs. In the presence of the other modifications, though, there are some interesting changes in cell biology such as morphology and signaling of downstream proteins, such as Erk1/2, c-Jun, and ATF-2 that are affected only in the presence of MAP3K1 loss. Taken as a whole, MAP3K1 loss alone may not be sufficient to induce carcinogenic changes, but in combination with other genetic changes may contribute to tumorigenicity.

Readers: Josh Lauring and David Valle

PREFACE

The following thesis summarizes my work as a graduate student working under Josh Lauring at the Johns Hopkins University School of Medicine Predoctoral Program in Human Genetics and is intended to be read by those wishing to gain insight on the role MAP3K1 may play in breast cancer. My sincerest gratitude is offered to Josh Lauring, without whom none of the following would be possible. I would also like to thank my family, living and passed, for the motivation to pursue such work as well as my friends for their support to continue.

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INTRODUCTION

Breast cancer is a common; with an estimated 234,190 new cases and 40,730 deaths in America in 2015, it ranks as the most prevalent (excluding skin cancer) and second deadliest cancer in women (Siegel, 2015). Breast cancer is a heterogeneous disease that is typically separated into four subtypes: luminal A, luminal B, HER2 amplified, and basal-like. Luminal A and B tumors both express the estrogen receptor and are therefore dependent upon estrogen for growth; luminal A differs from luminal B in having a lower expression of ki-67 and therefore a less proliferative phenotype (Inic, 2014). Anti-estrogens, such as Tamoxifen, can be used to great effect to block the hormone-dependent growth of luminal breast tumors. HER2-amplified tumors have an amplification or overexpression of the receptor tyrosine kinase ERBB2 gene. While more aggressive than luminal tumors, the recent advent of the monoclonal antibody trastuzumab (known as Herceptin) and targeted therapies that take advantage of antibody development have greatly improved the outcomes of patients with this breast cancer subtype. Basal-like breast cancers, also characterized as triple negative breast cancers, which do not express the estrogen receptor, progesterone receptor, or HER2 are difficult to treat and generally have the worst outcomes. Although these subtypes are broad in definition, researchers have been seeking to more acutely define breast tumors based on a number of molecular characteristics in hopes that targeted therapies can be used for such tumors to a maximum effect.

Within the past five years, one avenue researchers have been taking in order to fine tune the breast cancer subtypes has been through large-scale sequencing of tumors of the different subtypes. This has yielded a number of genes that appear mutated in breast cancer that had previously not been identified including ***AKT2***, ***CASP8***, ***NCOR1***, and ***MAP3K1*** (Stephens,

2012). As MAP3K1 has consistently arisen in such sequencing studies, and given its role as a kinase and the potential therapeutic options that could open, I decided to further investigate the role of MAP3K1 in breast cancer and the implications for treatment when it is lost.

Mitogen-activated protein (MAP) kinase kinase kinase 1, or MAP3K1, is a serine/threonine kinase that regulates MAP2K4 and JNK activation. MAP3K1 also has an E3 ligase domain that ubiquitylates c-Jun and ERK1/2. Serving as key node within the MAP kinase pathway, MAP3K1 has been associated with cell survival, migration, cell cycle regulation, and apoptotic regulation (Pham 2013). Knockout studies in mice have revealed that although MAP3K1 is not essential to life, intact and fully functioning MAP3K1 is necessary for complete embryonic eyelid closure (Jin, 2012). Further, although data available from The Cancer Genome Atlas Project (TCGA) suggest that with current treatment methods, patients with MAP3K1 loss do not appear to have a significantly better or worse prognosis compared to patients with normal expression of MAP3K1, there is not enough data to be conclusive (Cancer Genome Atlas, 2012). This is the case in all subtypes of breast cancer as well as in estrogen receptor positive tumors, in which ***MAP3K1*** mutations are most prevalent.

As stated above, recent large-scale sequencing studies have shown that MAP3K1 is mutated in a large proportion of breast cancers, specifically those in the luminal A subtype. A study undertaken by The TCGA performed whole-exome sequencing on 510 breast tumors from 507 patients to identify over 30,000 somatic mutations. They found that 8% of tumors across all breast cancer subtypes had ***MAP3K1*** mutations; when further characterized by subtype, this includes 13% of all luminal A tumors, 5% of luminal B tumors, 4% of HER2-amplified tumors, and none of the basal-like triple negative tumors (TCGA, 2012). Further, they also

identified that 12% of luminal A tumors contain likely inactivating mutations in either MAP3K1 or MAP2K4, which are in the same branch of the map kinase pathway. This is particular interesting as MAP3K1 directly activates MAP2K4.

Another study conducted by the Cancer Genome Project at the Wellcome Trust Sanger Institute similarly identified mutations in ***MAP3K1*** as potentially new drivers of breast cancer. They looked at the entire exomes of 100 breast tumors of which 79 were of the luminal, ER positive subtypes to identify 7,241 somatic point mutations (Stephens, 2012). They found somatic mutations in MAP3K1 in 6% of all breast tumors but mostly in the luminal subtypes, most of which were truncating and many of which occurred in a biallelic fashion resulting in loss of function.

A third study did performed whole genome sequencing on 46 estrogen-receptor positive breast tumors and whole exome sequencing on 31 in order to find out which genes are most associated with the different breast cancer subtypes as well as to discover the involvement in cancer of new genes. They found that ***MAP3K1*** mutation was most associated with luminal A breast tumors that were low-grade, and had low proliferation rates. They further identified thirteen mutations in their samples in the ***MAP3K1*** gene; most of these were nonsense and frameshift mutations that result in the predicted loss of function of that ***MAP3K1*** allele. They further noted a number of mutations in ***MAP2K4*** (the substrate of MAP3K1) that tended to also be found in luminal A breast tumors but not in those that already had mutations in ***MAP3K1***. This mutual exclusivity of ***MAP3K1*** and ***MAP2K4*** mutations suggests that the MAP kinase pathway specifically is related to luminal A breast cancer subtypes with ***MAP3K1*** mutations contributing the bulk of this effect. Building upon their initial findings, this group then investigated a validation set of 240 breast tumors and found 62

nonsynonymous mutations in either ***MAP3K1*** (52 mutations in 39 tumors) or ***MAP2K4*** (10 mutations) to makeup a total ***MAP3K1/MAP2K4*** mutation frequency of 15.5%. Of note, 13 of the tumors in this validation set had biallelic inactivation of ***MAP3K1*** further indicating its role as a tumor suppressor (Ellis, 2012).

A number of studies have also pointed to a potential connection of MAP kinase signaling and the PI3K/AKT pathway (Laprise, 2004). Specifically, PI3K has been shown to downregulate MAP kinase signaling. Investigations into PI3K signaling have shown mechanistically that activated PIK3CA, a component of PI3K, leads to breast cancer. Given that MAP3K1 loss is associated with breast cancer, it stands to reason that a potential mechanistic connection between activated PIK3CA and downregulated MAP3K1 may also exist in breast cancer. This is further supported in the TCGA study which found ***PIK3CA*** missense mutations and ***MAP3K1*** truncating mutations to coexist in a number of cancers (Cancer Genome Atlas, 2012)(Figure 1).

As ***MAP3K1*** tends to be mutated in a large proportion of luminal breast cancers, I decided to further investigate the role of MAP3K1 in breast cancer and its implications for treatment. While not estrogen-receptor positive, MCF-10A cells are epithelially-derived as luminal subtype breast tumors are. Specifically, though, estrogen-receptor positive breast cancer would be the best model for MAP3K1 loss as that seems to be the context of the majority of ***MAP3K1*** mutations. Although I took a number of avenues to investigate how MAP3K1 loss would affect breast cells, breast cancer cells, and treatment of such cells, for the sake of brevity only the most important of my findings follow.

METHODS AND RATIONALE

Cell lines

From the literature, ***MAP3K1*** appears to be a tumor suppressor gene that, in the context of breast cancer, mostly appears in estrogen-receptor positive tumors. Although it is estrogen receptor negative, the non-tumorigenic breast cell line, MCF-10A, was initially used to model MAP3K1 loss. MCF-10A is a widely used human breast epithelial cell line that is often used to model transformation. As it is non-tumorigenic, breast cancer researchers use it to make genetic alterations to the cells and investigate their effects as regards classic tumor hallmarks. Faster growth, abnormal morphology, or ability to invade a basement membrane and migrate may indicate that the genetic alterations that led to such phenotypes are involved in cancer. MCF-7 is a tumorigenic, estrogen-receptor positive breast cancer cell line that is derived from a luminal A tumor. MCF-7, while having multiple copies of ***MAP3K1***, expresses the gene normally (Figure 2). Modeling MAP3K1 loss in the MCF-7 cell line may show changes in its tumorigenicity or modulate its response to therapies.

MAP3K1 loss in MCF-10A cell line

MAP3K1 was targeted for deletion in MCF-10A cells by an adeno-associated-virus based method that selectively inactivated its two alleles (Figure 3). The targeting plasmid for the first allele was designed with homology arms flanking the second exon of ***MAP3K1*** with a 1 kilobase long region missing between the two homology arms. The homology arms directed the insertion of cre-lox sites that flanked an IRES-driven neomycin cassette which allowed for selection based on targeting. Following homologous recombination and cre-mediated recombination, the one kilobase long region between the two homology arms was excised resulting deletion of exon 2 and subsequent inactivation of that allele. The targeting

plasmid for the second allele similarly targeted exon 2, but in a different manner as using the same targeting vector for different alleles can result in retargeting of the first allele. The homology arms were designed to target the region deleted by the first targeting construct, with the IRES-neo cassette integrating within the second exon. Following homologous recombination and cre-mediated recombination, the loxP scar fell within the second exon and resulted in a frameshift with stop codons in all reading frames.

The two targeting plasmids for the 2 ***MAP3K1*** alleles were transformed into competent E. coli, purified via maxipreps, and sequenced to verify the plasmids. These were then transfected into HEK293T cells along with the associated plasmids coding for adeno-associated viral packaging with FuGENE 6 transfection reagent. Plasmids and the transfection reagent were diluted in serum-free, reduced growth medium, mixed, and added to HEK293T cells in T75 culture flasks. After 3 days incubation, cells were harvested, spun down, and subjected to freeze-thaws in order to liberate adeno-associated virus particles. The resulting mixture was then filtered to separate the virus particles from the cellular debris. The day prior to infecting MCF-10A cells with the virus that targeted the first ***MAP3K1*** allele, cells were seeded in T75 culture flasks to achieve 50% confluency in growth arresting medium. On the day of infection, medium was removed, 10mL of virus was added, and cells were allowed to incubate for 24 hours. After 24 hours, 10mL fresh culture medium was added to the T75 culture flasks and cells were again allowed to incubate for another 24 hours, at which point the medium was removed and replaced with fresh medium with G418 at 120 ug/mL for 1 week with medium replaced after 3 days. Clonal, single-allele ***MAP3K1*** knockout MCF-10A cells were isolated through single-cell dilution after PCR screening for targeted integration of the knockout construct on pools of cell colonies. Following PCR verification of clonal

lines in which the targeting vector had correctly inserted, Cre recombinase was added to incubating cells for one day to remove the vector. This process was repeated to knockout the second allele of ***MAP3K1*** in these cells. Immunoblotting was used to confirm the presence or absence of fully functional MAP3K1 protein.

MAP3K1 loss in MCF-7 cell line

Targeting ***MAP3K1*** in MCF-7 cells proved to be more difficult than in MCF-10A cells. For one, the tri-/tetra-ploid genome of MCF-7 necessitated multiple rounds targeting. Also, targeting efficientcy was reduced in those cells for unknown reasons. As such adeno-associated virus methods to target ***MAP3K1*** in MCF-7 cells were ineffective, resulting in incomplete disruption of ***MAP3K1***. Thus, I took an approach that utilized the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology. The CRISPR/Cas9 expression plasmid, LentiCRISPR, containing a puromycin resistance gene for selection as well as a promotor-driven cloning site were ordered from Addgene with short guide RNA (sgRNA) strands designed according to the tools provided by the Zhang lab at MIT (Hsu, 2013). 4 short guide RNAs (sgRNA) specific to ***MAP3K1*** were cloned cloned into the CRISPR/Cas9 expression plasmid. The four different plasmids each targeting a different area of ***MAP3K1*** were amplified in E. coli, purified through maxipreps, and transfected in HEK293T cells along with packaging and envelope plasmids using FuGENE 6 transfection reagent for lentiviral packaging. The day before transfection, HEK293T cells were seeded in 6-well culture plates such that they would reach 70% confluency the day of transfection. Just prior to transfection, the CRISPR/Cas9-sgRNA plasmids as well as both the packaging and envelope plasmids were diluted in serum-free, reduced growth medium and mixed with separately diluted transfection reagent before being added to HEK293T cells in

antibiotic-free medium. Lentivirus particles were harvested two days later for infection of MCF-7 cells. The four cell cultures then underwent selection with puromycin for three days and single-cell dilution in order to isolate independent, clonally-derived colonies. Twenty-five verified clonally-derived colonies were screened via Western blotting to confirm the absence of functional MAP3K1 protein.

Estrogen Receptor Expression in MCF-10A MAP3K1 knockout cell lines

While MCF-10A cell lines are a useful cell culture model to study genetic changes that may be important in the development of cancer and serve as a model for transformation, a potential drawback for the purposes of studying MAP3K1 is its lack of estrogen receptor alpha expression. As had been previously shown, MAP3K1 is primarily lost in luminal breast cancers in which the estrogen receptor is expressed. So while MAP3K1 loss in MCF-10A cells may elucidate the role of MAP3K1 in certain contexts, it may not correctly capture the role of MAP3K1 in its proper biological context. As such, expressing the estrogen receptor in the MCF-10A derived cell lines would better model MAP3K1 loss as it actually happens in breast cancer.

The Park lab has been an innovator in modeling estrogen dependent growth in MCF-10A cells. They have created an expression plasmid that, when transfected into estrogen-receptor negative cells, will express the estrogen receptor alpha, allow for stable selection, and convert the biology of the cells into one dependent upon estrogen for growth. The resulting MCF-10A cells that have been transfected with this plasmid and are newly dependent upon estrogen for growth have been termed ERIN (estrogen receptor in non-tumorigenic) cells. They found that these cells express the estrogen receptor alpha and are growth stimulated by 17-beta-estradiol in the absence of epidermal growth factor (Abukhdeir, 2006).

Further, growth of these cells in the presence of 17-beta-estradiol is blocked by selective estrogen receptor modulators, such as tamoxifen.

Using this same estrogen receptor expression plasmid, I transfected my MCF-10A MAP3K1 knockout cells, the MCF-10A ***MAP3K1*** heterozygous cells, and MCF-10A wild type cells in order to make them growth-dependent on estrogen using the FuGENE 6 transfection reagent. Cells were plated the day before transfection in 6-well culture plated such that they would around 70% confluent on the day of transfection. Just prior to transfection, transfection reagent was incubated with serum-free, reduced growth medium and mixed with diluted plasmids containing the estrogen receptor alpha expression plasmid in serum-free, reduced growth medium. This mixture was then added to the above-mentioned cells in antibiotic-free culture medium and allowed to incubate or 48 hours after which they were selected with G418 at 120 ug/ml. Estrogen receptor alpha expression was assayed via Western blotting.

PIK3CA activation in MCF-10A MAP3K1 knockout cell lines

Additionally, some previous studies have shown potential crosstalk between the MAP kinase and PI3K-Akt pathway in breast cancer. This is further supported by the fact that many breast cancers have been found to have mutations in both ***MAP3K1*** and ***PIK3CA*** (Figure 1). As such, double mutant cell lines were created in MCF-10A cells in which MAP3K1 was both knocked out as described above and an activating mutation in PIK3CA was knocked in. The Park lab has done extensive work characterizing MCF-10A cells in which PIK3CA has been activated via a mutation in either exons 9 or 20 (Gustin, 2009). Either an E545K mutation in exon 9 or H1074R mutation in exon 20 confer constitutive activation to PIK3CA. They found that MCF-10A cells in which PIK3CA had been activated exhibited transformed phenotypes, most notably growth factor-independent growth.

Further, the Park lab found that activation of PIK3CA led to downstream phosphorylation of MEK/ERK signaling, a pathway which could cross-talk with MAP3K1. Thus it is reasonable to hypothesize that there may be synergistic effects in tumorigenesis in losing MAP3K1 and activating PIK3CA.

Using the same constructs that the Park lab used in their paper, I set about activating PIK3CA through both the exon 9 and 20 mutations in both wild-type MCF-10A cells as well as two MCF-10A MAP3K1 knockout cell line. Both the E545K mutation in exon 9 and H1074R mutation in exon 20 constructs were transformed into competent E. coli, purified via maxipreps, and sequenced to verify the plasmids. These were then transfected into HEK293T cells along with the associated plasmids coding for adeno-associated viral processing with FuGENE 6 transfection reagent. Plasmids and the transfection reagent were diluted in serum-free, reduced growth medium, mixed, and added to HEK293T cells in T75 culture flasks. After 3 days incubation, cells were harvested, spun down, and subjected to freeze-thaws in order to liberate adeno-associated virus particles. The resulting mixture was then filtered to separate the virus particles from the cellular debris. The day prior to infecting MCF-10A cells and the derived MAP3K1 knockout MCF-10A cells with this virus, cells were seeded in T75 culture flasks to achieve 50% confluency in growth arresting medium. On the day of infection, medium was removed, 10mL of virus was added, and cells were allowed to incubate for 24 hours. After 24 hours, 10mL fresh culture medium was added to the T75 culture flasks and cells were again allowed to incubate for another 24 hours, at which point the medium was removed and replaced with fresh medium with G418 at 120 ug/mL for 1 week with medium replaced after 3 days. After this point, stable, clonally derived PIK3CA activating cells were isolated through single-cell dilution after PCR screening for homologous integration of the targeting

construct and verified by sequencing and growth in the presence and absence of epithelial growth factor.

MAP2K4 silencing in MCF-7 cell line

Given how closely within the MAP kinase network MAP3K1 and MAP2K4 are, and that mutations in either appear to be mutually exclusive and inactivating, I further set out to silence MAP2K4 expression in the MCF-7 cell line. The purpose of this was dual in nature: to initially see whether inactivating this node of the pathway would lessen the tumor phenotype of the cell lines and as a follow up, see if the resulting phenotype was similar to that seen when MAP3K1 expression was suppressed through CRISPR targeting. Expression constructs containing short hairpin RNA (shRNA) specific to ***MAP2K4*** were transfected into HEK293T cells using the FuGENE 6 transfection reagent alongside packaging and envelope plasmids for lentivirus packaging. Cells were plated the day before transfection in 6-well culture plates such that they would be around 70% confluent on the day of transfection. Just prior to transfection, transfection reagent was incubated with serum-free, reduced growth medium and mixed with diluted plasmids containing different shRNA's with the associated lentivirus plasmids in serum-free, reduced growth medium before being added to HEK293T cells in antibiotic-free medium. Lentivirus with each shRNA were harvested two days later and used to infect MCF-7 cells, after which they were incubated for 48 hours and assayed for MAP2K4 expression.

Phenotypic Assays

I carried out a number of assays to look into typical cancer-related phenotypes in order to investigate the effects of the above genomic modifications. Growth assays were carried out according to conditions called for by the specific cell lines being investigated. In the most

basic of cases, cells are to be seeded in either 6-well or 24-well culture plates at a low enough density to allow for growth by the end of the assay. If the effects of drugs are to be investigated, the following day medium is removed and replaced with drug containing medium. Cells are counted using a ViCell cell counter and normalized to a control (no genetic modification, no treatment, etc.).

Matrigel morphogenesis assays were carried out in order to investigate the effects of the above genetic modifications on the resulting cell line morphology in a simulated extracellular matrix. While the normal, non-tumorigenic MCF-10A cell line develops into well-formed acini, transformed lines tend to form unique structures such as cellular extensions, interconnected lobes, and large cellular masses. Matrigel morphogenesis assays were setup in 8-chamber culture slides. Thawed Matrigel is initially coated onto the bottom of the slides and allowed to set. Harvested cell lines are then suspended in Matrigel and seeded into each well of the chamber slide at a density of 1,000 cells/well. Culture medium is then layered on top. The slides are allowed to incubate for up to 3 weeks to allow for enough growth with the top layer of medium changed every 5 days.

A common method of visually viewing cell density is through staining with crystal violet. Crystal violet is a cellular stain that will be taken up only by viable cells. An added benefit to this technique is that crystal violet also fixes the cells, allowing for a visual comparison years into the future. A 0.005% crystal violet solution is created by dissolving 0.5 grams of crystal violet in 25mL methanol and 75mL water. Medium is removed from cells and the crystal violet solution is added in a volume sufficient to just cover the cells. The cells are incubated with the solution at room temperature for 30 minutes, after which point cells

are washed with DPBS to remove excess stain and allow visualization. The resulting stained and fixed cells can be visually compared to others stained similarly to view differences in growth in different media as well as put into solution to allow for spectrophotometric comparisons.

Western blotting was used to investigate downstream effects of the genetic editing and drug studies. For this, cells were seeded between 250,000 and 500,000 cells in a 6-well culture plate and allow for growth for a specified time point, often for 24 to 48 hours. Cells were cultured overnight in an experimentally relevant medium (with or without drug or growth factors) and lysates made the next day. To collect whole cell lysates, media would be aspirated from the wells, rinsed with DBPS, and a sufficient mixture of 10% 2-mercaptoethaol/2X Laemmli sample buffer solution added to cover the cells. Cells would then be scraped into a collection tube and boiled at 100 degrees Celsius for 10 minutes. Total protein concentration would be quantified via the Pierce 660nm protein assay reagent in order to normalize the total protein load in the gel. Lysates were run on 4-12% Bis-Tris precast gels for sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred to Invitrolon PVDF, 0.45 μm pore size membranes after which they were blocked with a 5% milk/0.5% Tween 20/Tris-buffered saline solution. Blocked membranes were probed for a protein of interest through incubation at 4 degrees Celsius overnight in a solution of antibody diluted in 5% milk/0.5% Tween 20/Tris-buffered saline. After rinsing the membrane, diluted secondary antibody specific to the primary antibody was incubated at room temperature for one hour in a 5% milk/0.5% Tween 20/Tris-buffered saline solution. After rinsing, the membrane was briefly incubated in Perkin Elmer enhanced chemiluminescence western blotting substrate and visualized through exposure to film and subsequently developed.

Densitometric calculations were derived from ImageJ software and normalized by comparison to housekeeping protein expression levels.

RESULTS AND DISCUSSION

Creation of MAP3K1 knockout cell lines and other cell culture models

MAP3K1 loss in MCF-10A cell line

In all, 4 MAP3K1 knockout cell lines were successfully derived from MCF-10A wild-type cells (Figure 4). Two independent clonal cell lines were derived from the wild-type MCF-10A cells in which one allele had been inactivated through the first targeting vector, called 13-B1 and 13-A2. From each of these, two more independent cell lines were clonally derived in which both alleles had been inactivated: 13-B1 yielded 5C95 and 6H92 while 13-A2 yielded 5D95 and 6D91. Additionally, two cell lines were derived from 13-B1 which had undergone the targeting and neomycin selection process but had non-homologous integration of the targeting construct, called 5C3 and 6H3. 5C3 and 6H3 had thus undergone the same procedures as the MAP3K1 knockout cell lines but retained one active allele; as such they can be used as controls for the MAP3K1 knockout cell lines as well as used to investigate the effect of MAP3K1 haploinsufficiency compared to losing all copies of MAP3K1. Immunoblotting confirms that full length MAP3K1 is absent from the four MAP3K1 knockout cell lines derived from MCF-10A, 5C95, 6H92, 5D95, and 6D91 (Figure 5). The two single allele knockout cell lines, 5C3 and 6H3, retain full-length MAP3K1, although perhaps not to the extent that wild-type MCF-10A cells do.

The initial classic tumor phenotype I wanted to investigate in these derived cell lines was their growth kinetics (Figure 6). Specifically, I

wanted to know whether MAP3K1 loss in this model induced growth factor independence, one of the hallmarks of tumorigenicity. MCF-10A cells normally require epidermal growth factor (EGF) at a concentration of 20 ng/mL but can grow slowly in 1% of that. Growth is arrested in the absence of EGF. In the absence of EGF, none of the derived cell lines showed any growth alone or in comparison to wild-type MCF-10A cells (data not shown). This is regardless of how many days cells were allowed to grow or the initial seeding density. In low EGF conditions of 0.2 ng/m (representative experiment shown in Figure 6), no cell line outperformed the others in terms of growth consistently. Allowing growth to occur for eight days in this low EGF condition did not show any appreciably different growth kinetics. At physiological EGF levels of 20 ng/mL, the MCF-10A derived MAP3K1 knockout, single-allele knockout, and wild-type MCF-10A cells consistently grew at similar rates. These experiments demonstrate that MAP3K1 loss does not have any effect on the growth kinetics in the MCF-10A non-tumorigenic breast cell line.

Similar to looking into the growth kinetics in culture, I wanted to know what the growth would be like in a three-dimensional culture model. Specifically, a Matrigel extracellular matrix culture was used to mimic the biological microenvironment of the tumor to see the morphology my MCF-10A derived MAP3K1 cell lines would take. In comparison to wild-type MCF-10A cells, neither the two single-allele MAP3K1 knockout cell lines nor the four complete MAP3K1 knockout cell lines demonstrated any morphological differences compared to their wild-type MCF-10A counterparts (data not shown). This was true regardless of whether they were cultured in physiologic levels of EGF (20 ng/mL) or low levels of EGF (0.2 ng/mL), or regardless of how long they were allowed to stay in culture. MAP3K1 loss, then, had no effect on the morphology of the MCF-10A non-tumorigenic breast cell line in Matrigel.

The effects of commonly used chemotherapeutic agents on the derived MCF-10A MAP3K1 knockout cell lines has important clinical implications. Should MAP3K1 loss sensitize the MCF-10A derived cell line, it may be an indication that women with such mutations would benefit from a given treatment. I looked primarily into two chemotherapeutics: nocodazole and paclitaxel. Nocodazole is a drug that interferes with microtubule polymerization; prolonged exposure to this and at high enough concentrations causes cell death due to growth arrest during mitosis. Paclitaxel also has its effects on microtubules; however, it prevents their degradation and eventually leads to cell death from their accumulation. Paclitaxel is widely used clinically to treat breast cancer, whereas nocodazole is not a clinical drug. In other settings, MAP3K1 has been shown to be required for apoptosis induced by microtubule disruption (Johnson, 1999). Figure 7 shows the growth of the different derived cell lines when exposed to the two above drugs. Blue lines represent MCF-10A wild-type cells; the green lines represent the average of the 2 MCF-10A derived single-allele knockout cell lines 5C3 and 6H3; the red lines represent the average of the average of 4 MCF-10A derived, MAP3K1 knockout cell lines 5D95, 6H92, 6D91, and 5C95 with each cell line treated in triplicate. Regardless of whether the cell lines were treated with nocodazole (Figure 7a) or Paclitaxel (Figure 7b), cell lines did not show an appreciable difference in growth indicating that MAP3K1 does not sensitize the MCF-10A non-tumorigenic breast cell line to either of these agents.

MAP3K1 loss in MCF-10A cells did, however, have some effect on downstream protein signaling through the MAP kinase pathway that may have potential relevance for tumor behavior, specifically in the context of nocodazole treatment (Figure 8). 2 MCF-10A derived MAP3K1 knockout cell lines (6D91 and 5C95), 1 MCF-10A derived single-allele MAP3K1 knockout

cell line (5C3) and wild-type MCF-10A cells were treated with nocodozole and a DMSO vehicle control at 45 nM. Cell lysates were collected at three time points: 1 hour, 8 hours, and 24 hours and immunoblotted for phospho-c-Jun as well as the loading control marker GAPDH. Phospho-c-Jun induction as a result of nocodazole treatment may be decreased in the MAP3K1 knockout cell lines compared to their wild-type and single-allele knockout controls.

MAP3K1 loss in MCF-7

Of the twenty-five screened MCF-7 colonies transfected with the CRISPR-MAP3K1-KO plasmids, at least two single-cell derived clones from each sgRNA targeting construct had no expression of functional MAP3K1 protein as assayed via western blotting. Initial growth assays suggest that there was no significant difference in growth between any of the 4 ***MAP3K1*** knockout clones when compared to either an empty vector control or parental MCF-7 cells (data not shown).

Estrogen Receptor Expression in MCF-10A MAP3K1 knockout cell lines

Of the MCF-10A derived ***MAP3K1*** knockout cell lines I had been working with, I was able to induce estrogen receptor expression in four clones across the three main categories (Figure 9a). Those in which estrogen receptor expression was induced include two parental MCF-10A cell lines, two heterozygous, single-allele knockout cell lines (of the 5C3 lineage), and three from the two-allele knockout cell lines (one of the 6H92 lineage and two of the 6D91 lineage). Perhaps owing to their clonal nature, estrogen receptor expression was highly variable across the different derived lines and even within clones having the same parental cell line (Figure 9b).

Wild-type MCF-10A cells normally require growth factors for growth. This is altered with the expression of the estrogen receptor, which allows the cells to grow dependent on estrogen instead. Figure 10 shows a selection of estrogen receptor expressing cell lines (boxed in green) as well as those that underwent transfection of the estrogen receptor expression plasmid but did not show any appreciable expression levels (boxed in red) which have been stained with crystal violet after 17 days in culture. In the absence of estrogen, no cell line exhibited any growth. For those that show positive estrogen receptor expression (green boxes), growth is appreciably better in 17-beta-estradiol than in the ethanol vehicle control. The growth seen in the estrogen receptor expressing cell lines is neutralized in the presence of the selective estrogen receptor modifiers tamoxifen and faslodex. There does not appear to be any differential effect of 17-beta-estradiol on these cells with respect to MAP3K1 knockout status. This suggests that any growth differential is due to factors other than ***MAP3K1*** status.

The most significant difference regarding ***MAP3K1*** loss in estrogen receptor expressing cells has to do with signaling downstream of MAP3K1 (figure 11). In the absence of epithelial growth factor, ***MAP3K1*** knockout cells do not exhibit Erk1/2 activation whereas the parental MCF-10A cell lines that express estrogen receptor maintain the ability to activate Erk1/2. Considering the role of the extracellular signal-regulated kinases, Erk1/2, in carcinogenesis, this finding warrants further investigation.

PIK3CA activation in MCF-10A MAP3K1 knockout cell lines

A number of double mutant cell lines were derived in which ***MAP3K1*** had been knocked out and PIK3CA had been activated (Figure 12a). ***PIK3CA*** had been activated both through the exon 9 and exon 20 mutations with most

phenotypic workup focused on the exon 9 mutation. In the absence of growth factors, only those cell lines with activated PIK3CA (exon 9) exhibit growth. However, ***MAP3K1*** knockout status does not appear to affect this growth appreciably, as shown in the similar cell densities of the crystal-violet stained wells (Figure 12b). As such, growth differences in these cell lines are most likely either related to their clonal nature or due to PIK3CA activation solely.

There does, though, appear to be a difference in these double mutant cell lines' ability to form acini in Matrigel (Figure 13). The acini formed by the double mutant cells in which MAP3K1 was knocked out and PIK3CA activated have a much more abnormal morphology compared to either mutation alone. This suggests the two mutations cooperate or synergize to alone cause aberrant morphology.

There were some interesting findings concerning downstream signaling proteins, though (Figure 14). ***MAP3K1*** loss in MCF-10A cells appears to be associated with increased ATF-2 (activating transcription factor 2) activation, decreased p38 (a mitogen-activated protein kinase) activation, and increased c-Jun activation. Such findings may explain the tumorigenic qualities of MAP3K1 loss and deserve further research.

MAP2K4 silencing in MCF-7 cell line

A number of clones were derived from MCF-7 cells that exhibited markedly reduced expression of MAP2K4. Further workup of these cell lines was not performed.

CONCLUSIONS

While there may be some effect of MAP3K1 loss in the development of breast cancer, I was not able to show definitively how or why that may be. The high prevalence of women with breast cancer having MAP3K1 loss is not likely due to it being a passenger mutation in the carcinogenesis process. This is based on previous genome-wide breast cancer studies consistently finding an association with disease as well as its mutual exclusivity with MAP2K4 which is not common in other cancer types. More likely is that MAP3K1 may exert its effects in ways not investigated or yet to be identified. For one, the models of MAP3K1 loss discussed above do not model all aspects of tumor biology. The tumor microenvironment, tumor-stroma interactions, and other factors associated with systems biology were not able to be investigated in such cell culture models. Further, it may be that MAP3K1 is and its loss are important in the context of the breast, a more complex a organ than is able to be modeled accurately in cell culture. MAP3K1 and its loss may also be significant in allowing for an ideal environment for tumorigenesis. The assays employed above were used to elucidate the role MAP3K1 in tumorigenicity, but would miss its role in tumorigenesis. The multiple changes in signaling pathways as a result of MAP3K1 loss, however, do demonstrate that further investigation is needed to identify its role in breast cancer.

FIGURES

Figure 1. Reproduction of Figure 1 from TCGA 2012 article showing class of mutations in highly mutated genes in breast cancer separated by subtype. This graphic shows significantly mutated genes within breast cancer subtypes (225 luminal A tumors, 126 luminal B tumors, 57 HER2-enriched tumors, 93 basal-like tumors). Note that ***MAP3K1*** mutations mainly manifest as a truncation mutation as opposed to a missense mutation. This would likely result in inactive protein. ***MAP3K1*** mutations occur in 8% of all breast cancers but are enriched in Luminal A subtypes in which they occur at a rate of 13%. In MAP3K1 and MAP2K4, proteins which occupy two contiguous steps in the MAP kinase pathway, mutations appear to be mutually exclusive, occurring in one or the other. Further, a significant percentage of MAP3K1 mutations occur in luminal A tumors in which there is also a PIK3CA mutations (The Cancer Genome Atlas, 2012). This figure is reproduced under the Creative Commons Attribution-Non-Commercial-Share Alike license which allows academic reproduction of such material.

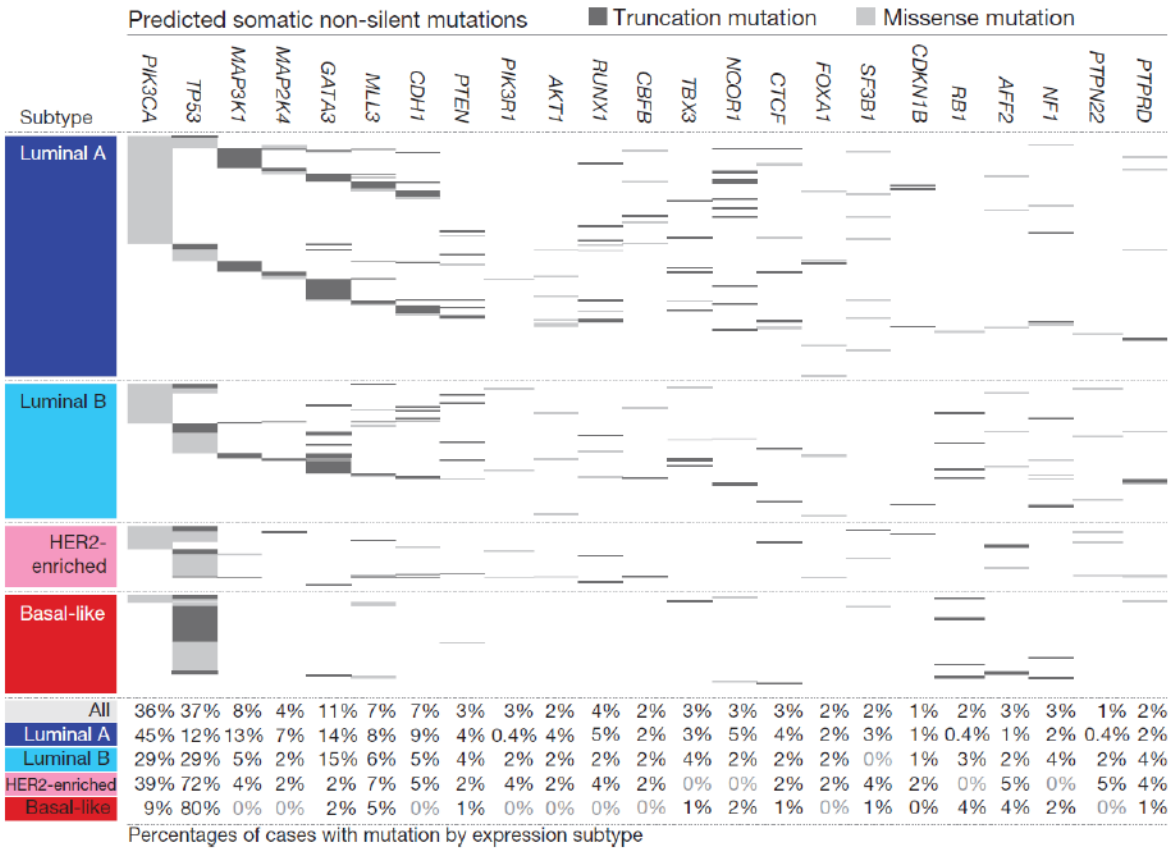
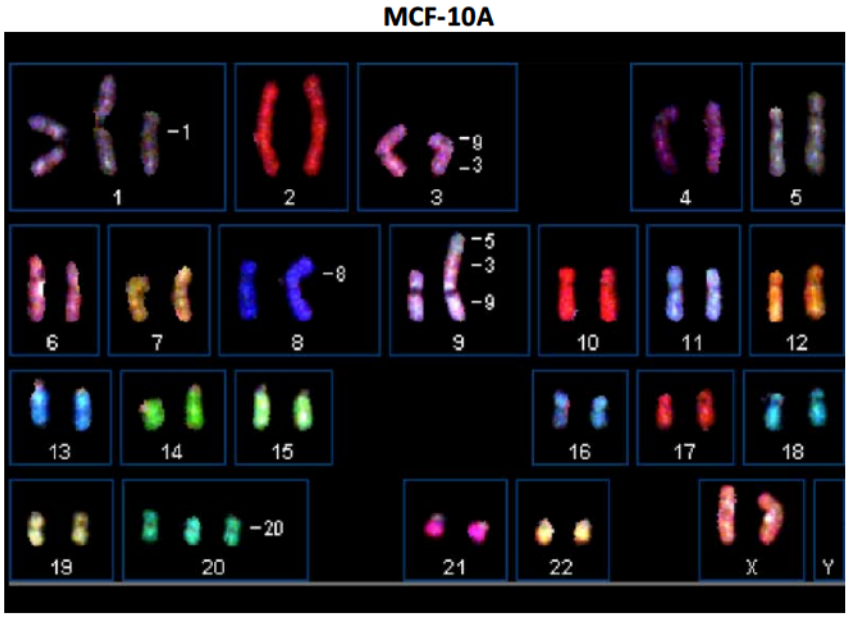


Figure 2. Comparison of the MCF-10A and MCF-7 cell line karyotypes. MCF-10A cells (top) are a non-tumorigenic, epithelial derived, mostly diploid cell line. With the exception of a few transformations, the t(3;9) which confers immortality for one, MCF-10A is a mostly diploid cell line. MCF-7, in contrast is a tri/tetra-ploid, estrogen receptor positive, growth factor independent breast cancer cell line. (Cowell, 2005; NCBI SKY/M-FISH & CGH Database, http://www.ncbi.nlm.nih.gov/sky/skyquery.cgi)



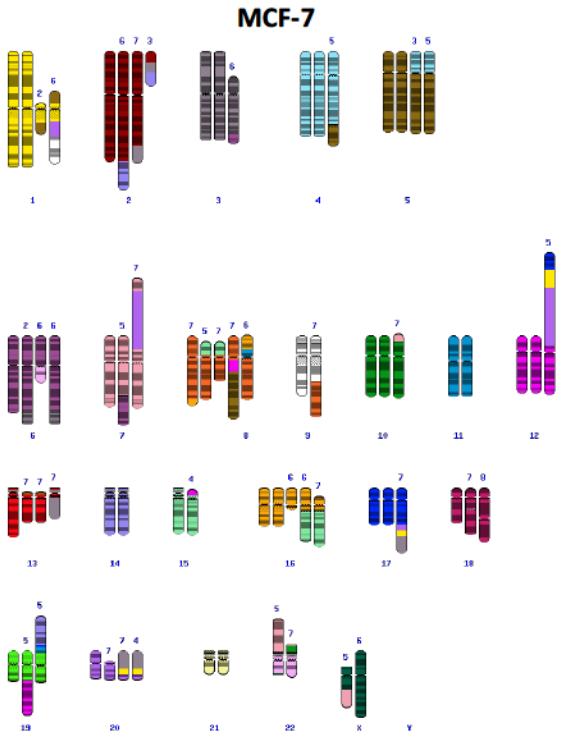


Figure 3. MAP3K1 targeting strategy in MCF-10A. MCF-10A cells have 2 alleles of MAP3K1; two targeting vectors were designed to each allele. The first allele was inactivated through the deletion of exon 2 while the second allele was inactivated through the interruption and subsequent creation of stop codons within exon 2.

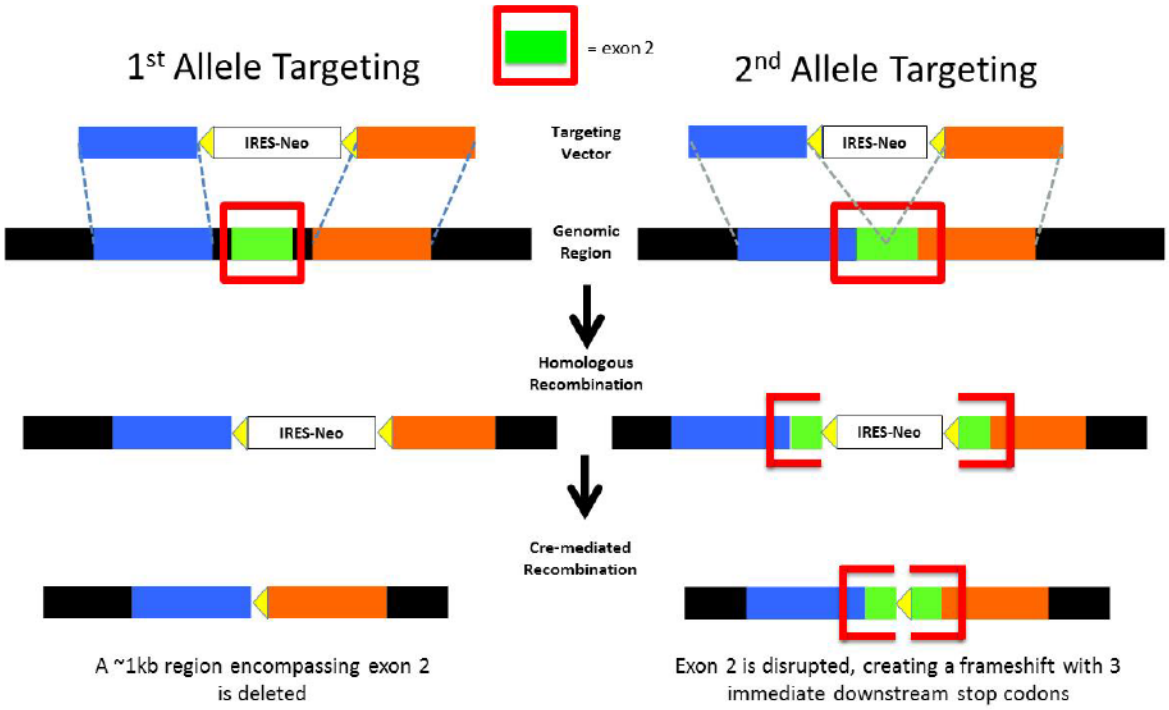


Figure 4. MCF-10A MAP3K1 knockout cell lines. The following are clonally-derived, independently isolated cell lines from MCF-10A in which either a single allele or both alleles of MAP3K1 has been inactivated. The green member on top with "+/+" is MCF-10A wild-type cell line from which all the following members were derived. The yellow members on the second tier with "+/-" are cell lines in which one allele has been inactivated via the first allele targeting vector. The orange members with "+/-" on the bottom tier were derived from the 13-B1 cell line and similarly have one allele inactivated. However, these cell lines have also undergone targeting via the second allele targeting vector but underwent nonspecific integration of the targeting vector. The red members with "-/-" on the bottom tier were derived from either single allele inactivated cell lines and have both of their MAP3K1 alleles inactivated.

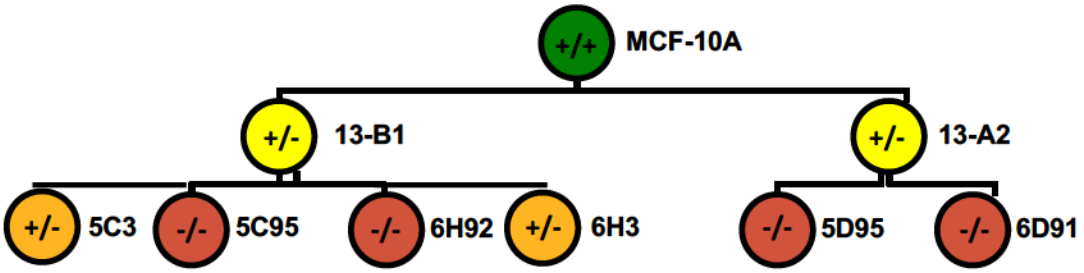


Figure 5. Verification of MCF-10A MAP3K1 knockout cell lines by western blotting. The knockout cell lines derived from MCF-10A cells have no full-length MAP3K1 protein. The single-allele inactivation cell lines retain fully functional MAP3K1.

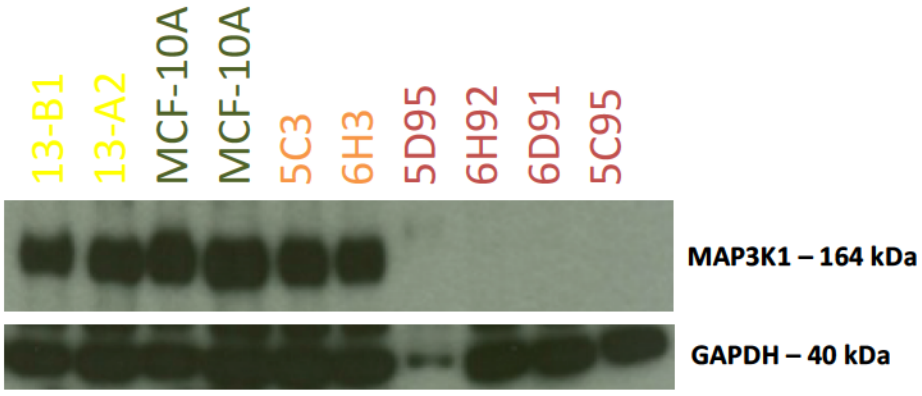
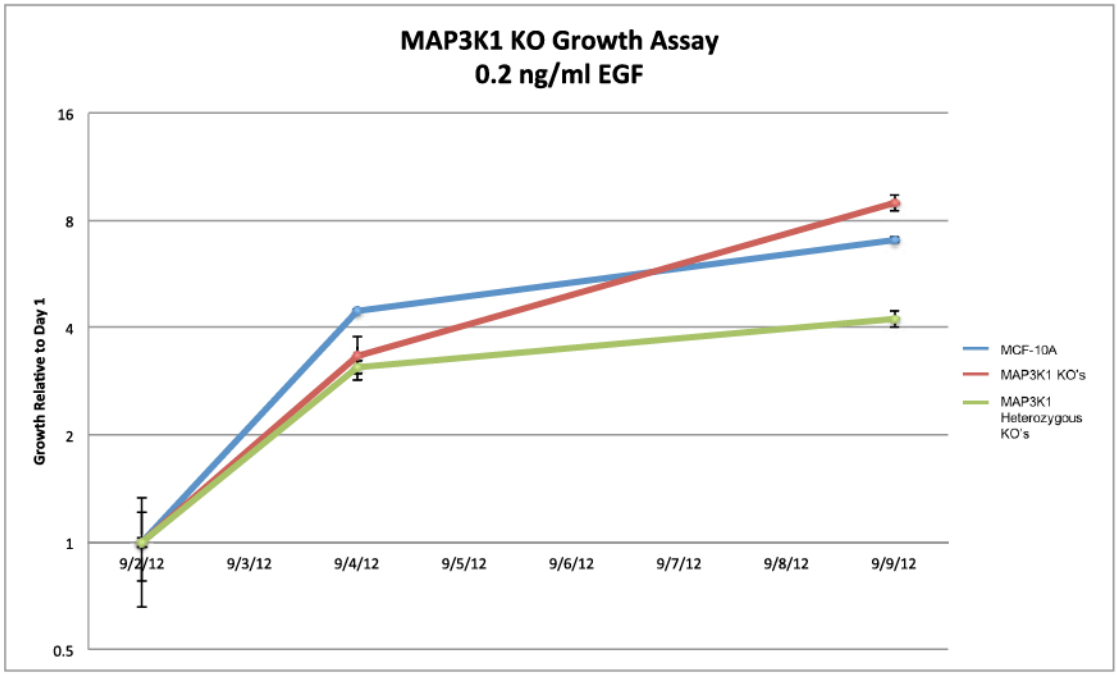


Figure 6. Growth of MCF-10A derived MAP3K1 knockout and single-allele knockout cell lines in low and physiological growth factor concentrations. Blue lines represent MCF-10A wild-type cells; the green line represents the average of the 2 MCF-10A derived single-allele knockout cell lines 5C3 and 6H3; the red line represent the average of the average of 4 MCF-10A derived, MAP3K1 knockout cell lines 5D95, 6H92, 6D91, and 5C95. Each cell line was treated in triplicate; this experiment was repeated to confirm results. In both growth conditions there is no appreciable, biological difference between the different cell lines.



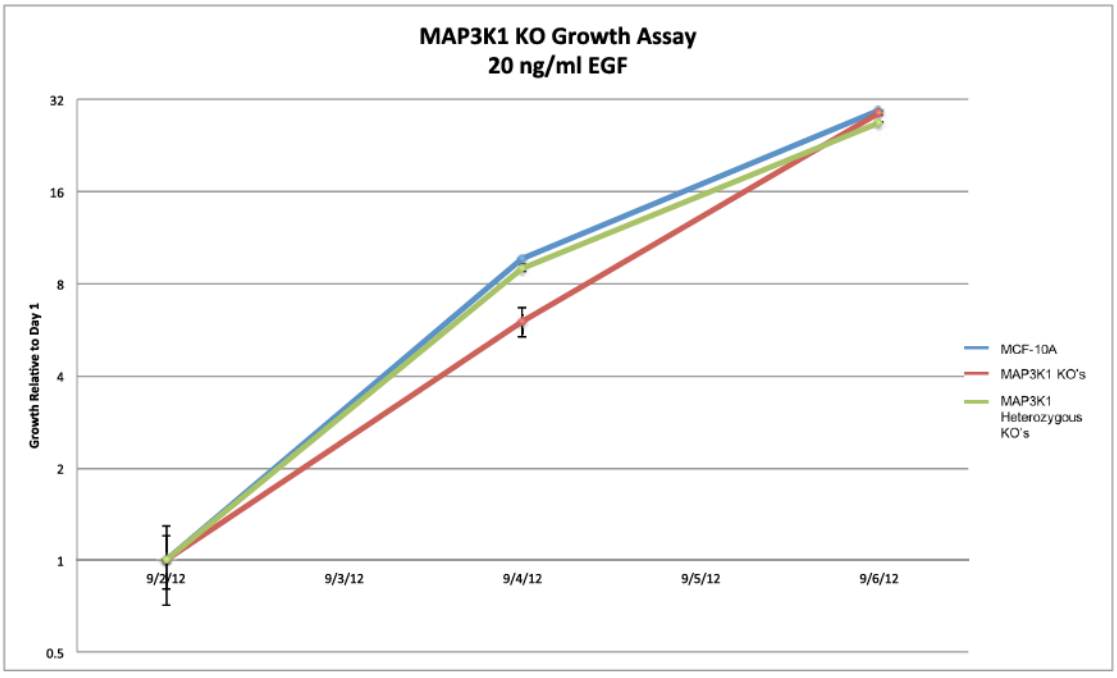
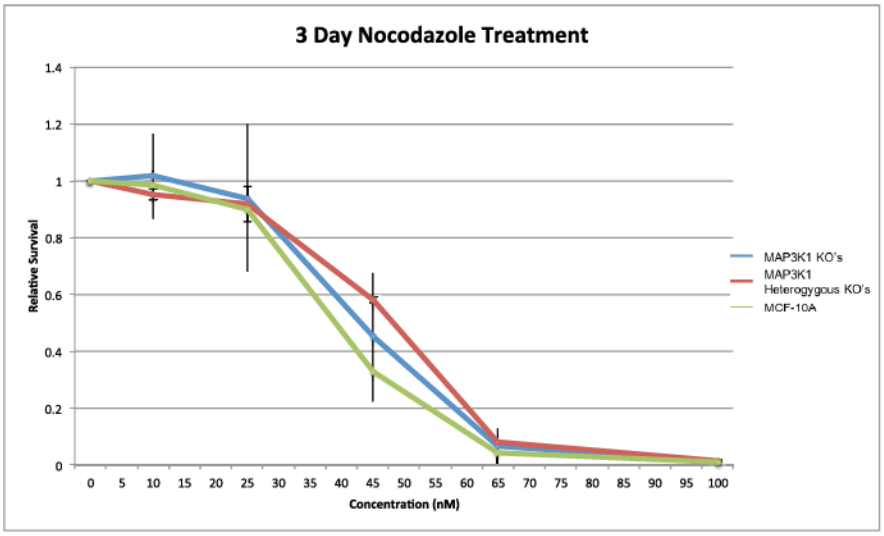


Figure 7. Effects of nocodazole and paclitaxel on growth in MCF-10A derived MAP3K1 cell lines.

a. MCF-10A wild-type cell line, two MCF-10A derived MAP3K1 single-allele knockout cell lines 5C3 and 6H3, and the four MCF-10A derived, MAP3K1 knockout cell lines 5D95, 6H92, 6D91, and 5C95 were exposed to nocodazole at varying concentrations. Cells were exposed to the drug for three days; lines represent the average of the stated cell line types. There was no appreciable difference in growth or survival in the drug across the different cell lines.



b. MCF-10A wild-type cell line, two MCF-10A derived MAP3K1 single-allele knockout cell lines 5C3 and 6H3, and the four MCF-10A derived, MAP3K1 knockout cell lines 5D95, 6H92, 6D91, and 5C95 were exposed to paclitaxel at varying concentrations. Cells were exposed to the drug for three days; lines represent the average of the stated cell line types. There was no appreciable difference in growth or survival in the drug across the different cell lines.

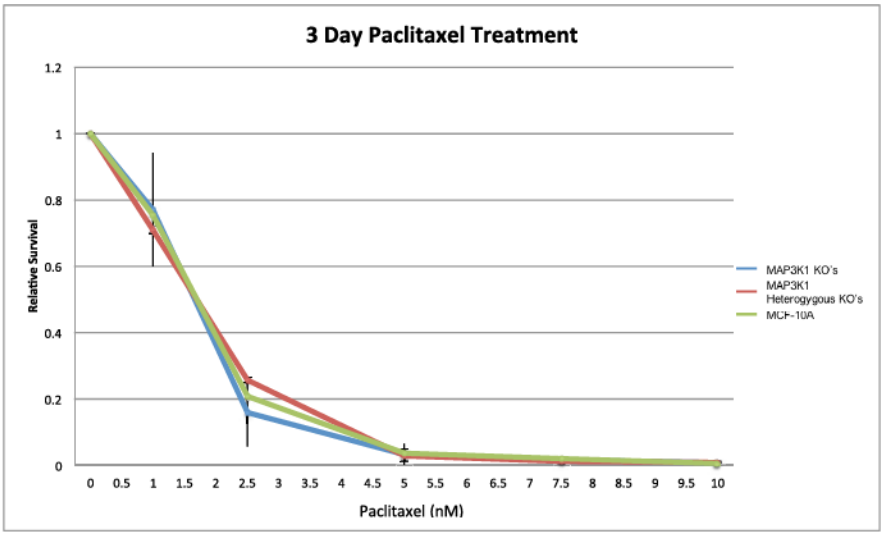


Figure 8. Effects of nocodazole on the steady-state levels of downstream proteins in MCF-10A derived MAP3K1 cell lines. 2 MCF-10A derived MAP3K1 knockout cell lines (6D91 and 5C95), 1 MCF-10A derived single-allele MAP3K1 knockout cell line (5C3) and wild-type MCF-10A cells were treated with nocodozole and a DMSO vehicle control at 45 nM. Cell lysates were collected at three time points: 1 hour, 8 hours, and 24 hours and immunoblotted for phospho-c-Jun as well as the loading control marker GAPDH. Phospho-c-Jun induction as a result of nocodazole treatment may be decreased in the MAP3K1 knockout cell lines compared to their wild-type and single-allele knockout controls.

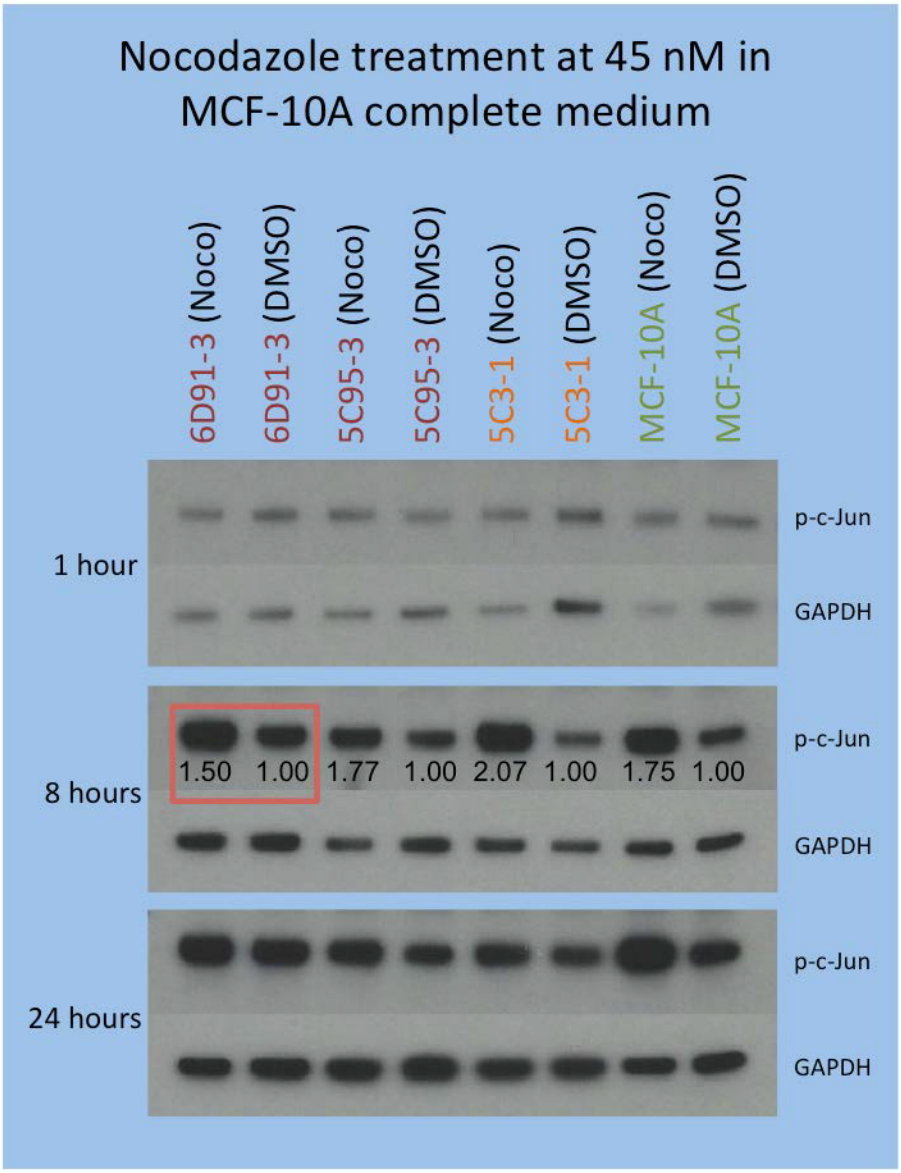
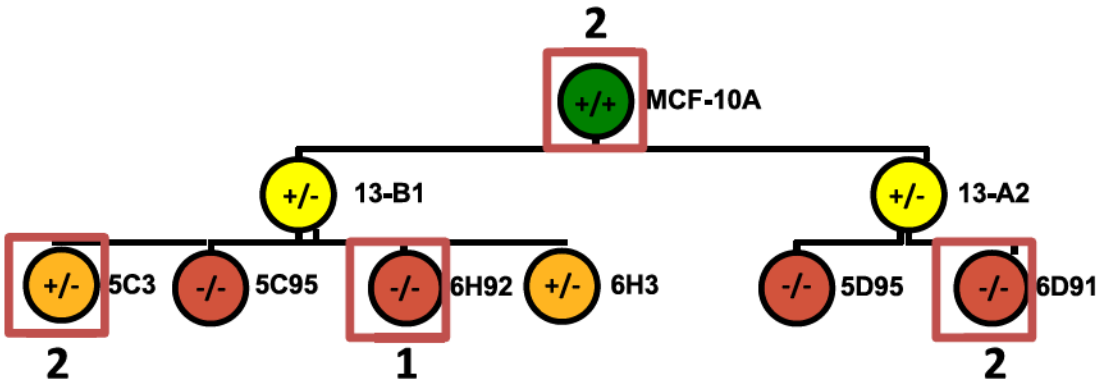


Figure 9. Estrogen Receptor Expression in MCF-10A *MAP3K1* knockout cell lines.

a. The derivation scheme and numbers of the MCF-10A *MAP3K1* knockout cell lines underwent transfection of ER alpha. 2 wild-type MCF-10A cell lines, 2 single-allele inactivation cell lines, and 3 *MAP3K1* knockout cell lines were successfully transfected with the ER expression plasmid.



b. Representative verification of estrogen receptor expression in MCF-10A MAP3K1 knockout cell lines by western blotting.

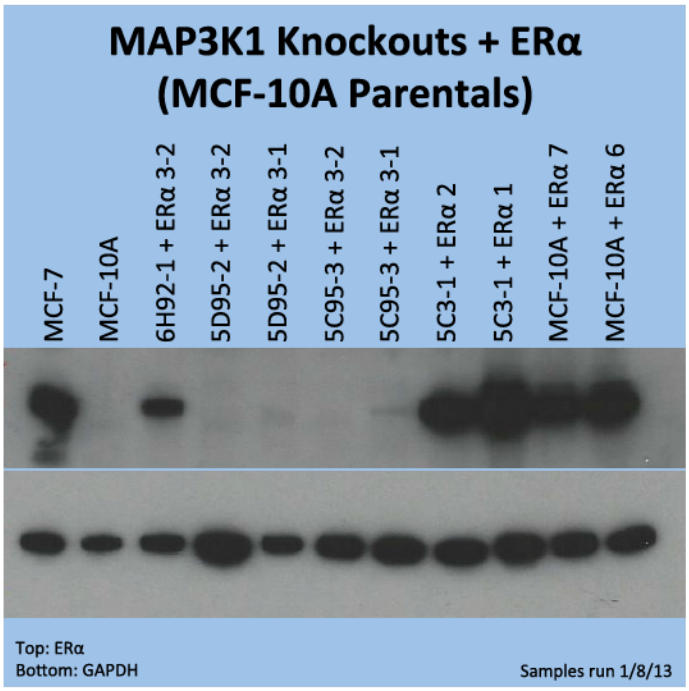


Figure 10. Effects of estrogen receptor expression in MCF-10A MAP3K1 knockout cell lines. Wild-type MCF-10A cells normally require growth factors for growth. This is altered with the expression of the estrogen receptor which allows the cells to grow dependent on estrogen. Shown below are a selection of estrogen receptor expressing cell lines (boxed in green) as well as those that underwent transfection of the estrogen receptor expression plasmid but did not show any appreciable expression levels (boxed in red) which have been stained with crystal violet after 17 days in culture. In the absence of any estrogen, no cell line exhibited any growth. For those that show positive estrogen receptor expression (green boxes), growth is appreciably better in 17-beta-estradiol than in the ethanol vehicle control. The growth seen in the estrogen receptor expressing cell lines is neutralized in the presence of the selective estrogen receptor modifiers tamoxifen and faslodex. There does not appear to be any differential effect of 17-beta-estradiol on these cells with respect to MAP3K1 knockout status.

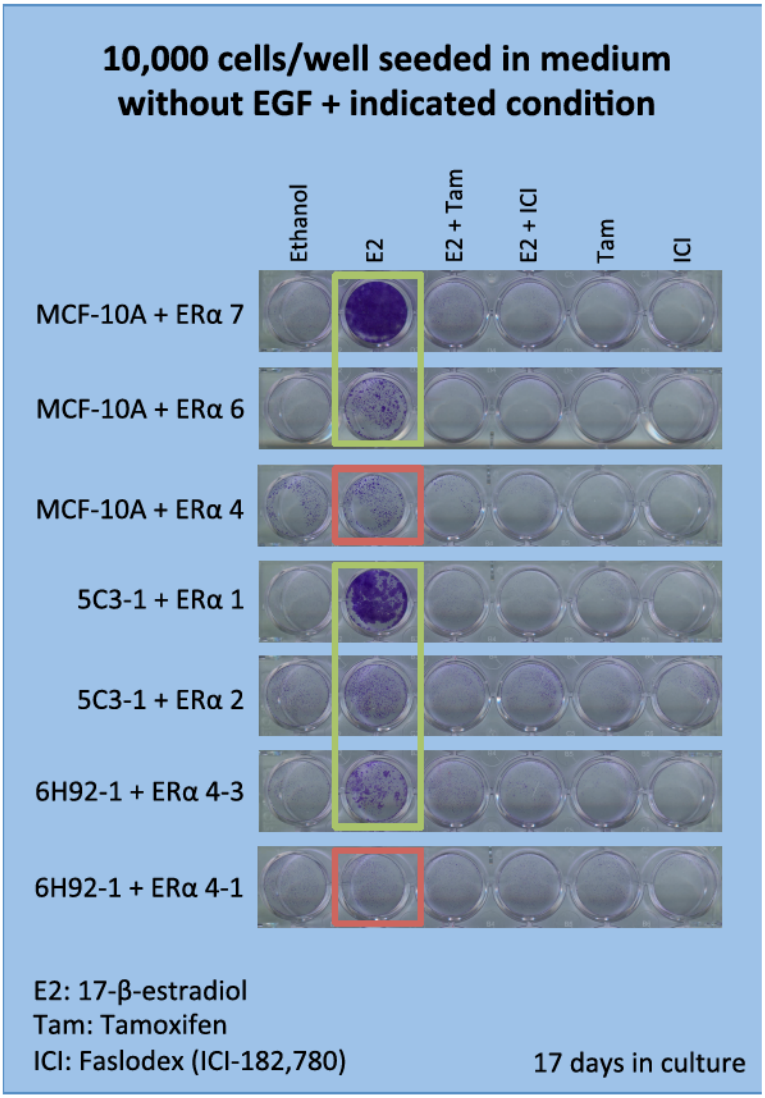


Figure 11. Downstream signaling of MAP3K1 in MCF-10A derived, estrogen receptor expressing cell lines with either MAP3K1 knocked out (left, red) or with fully active MAP3K1 (right, green). In the absence of epithelial growth factor, MAP3K1 knockout cells do not exhibit Erk1/2 activation whereas the parental MCF-10A cell lines that express estrogen receptor maintain the ability to activate Erk1/2.

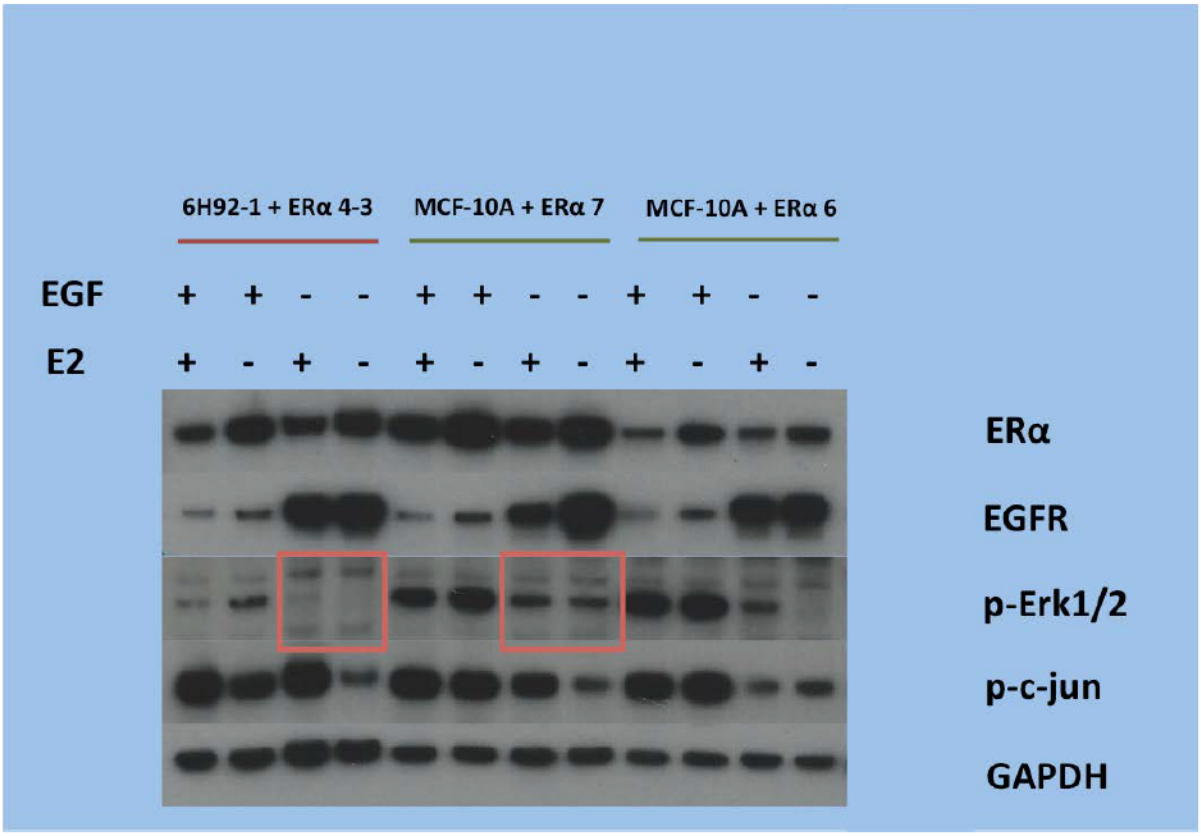
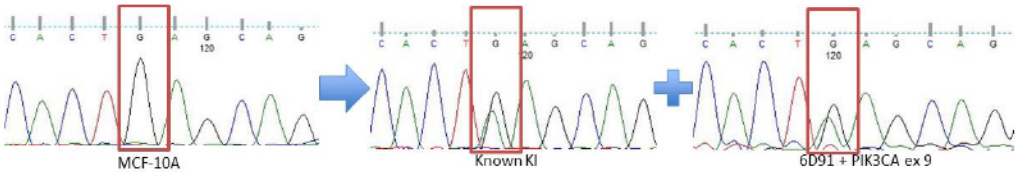


Figure 12. PIK3CA activation in MCF-10A MAP3K1 knockout cell lines. Knockin of the activating PIK3CA hotspot mutations in exons 9 and 20 were verified through sequencing and growth patterns without growth factors. 2 of the MCF-10A MAP3K1 knockout cells lines, 6H91 and 6D91, underwent targeted activation of PIK3CA. 2 clones from each of these cell lines each had PIK3CA activated through either the exon 9 or exon 20 mutation. Additionally MCF-10A wild-type cells underwent PIK3CA activation via mutation of both exons 9 and 20.

a. Shown here is a representative G>A mutation in exon 9 of PIK3CA in wild-type MCF-10A cells. The first panel is a chromatogram of wild-type MCF-10A cells showing a homozygous G at this location. The middle panel shows a chromatogram of a previously created and verified PIK3CA knock-in activating mutation in exon 9 in which one allele underwent a G>A mutation. The last panel shows a chromatogram from one of my MCF-10A MAP3K1 knockout cell lines in which PIK3CA has been mutated from G>A in one allele similar to what was seen in the previously verified cell line.



b. Representative crystal violet stained plate in which two of the MCF-10A MAP3K1 knockout cell lines (6H92 and 6D91) with activated PIK3CA (exon 9) as well as MAP3K1 knockout cell lines in which underwent targeting of PIK3CA but did not exhibit PIK3CA activation (labeled RI), MCF-10A wild-type cells, 6D91 & 6H92 MAP3K1 knockout cell lines without activated PIK3CA, and MCF-10A with activated PIK3CA (exon 9) (labeled 512). In the absence of growth factors, only those cell lines with activated PIK3CA (exon 9) exhibit growth. MAP3K1 knockout status does not appear to affect this growth appreciably.

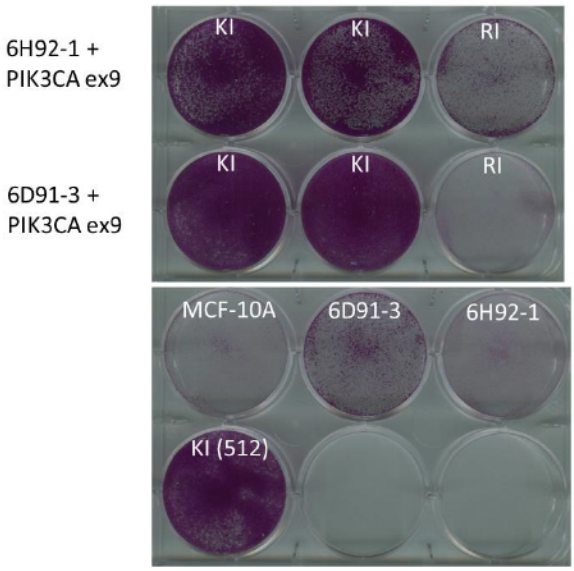


Figure 13. Microscopic images of Matrigel morphogenesis assay (representative images). All images are taken at a 4X magnification after being in Matrigel culture for 21 days. While the morphology of the wild-type parental MCF-10A cell line is similar to that of either MAP3K1 knockout cells or PIK3CA activated cells alone, having both mutations present appears to result in a somewhat abnormal morphology with less defined, less rounded acini being common.

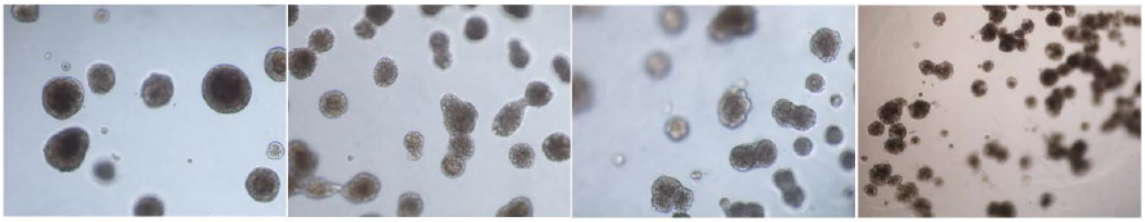
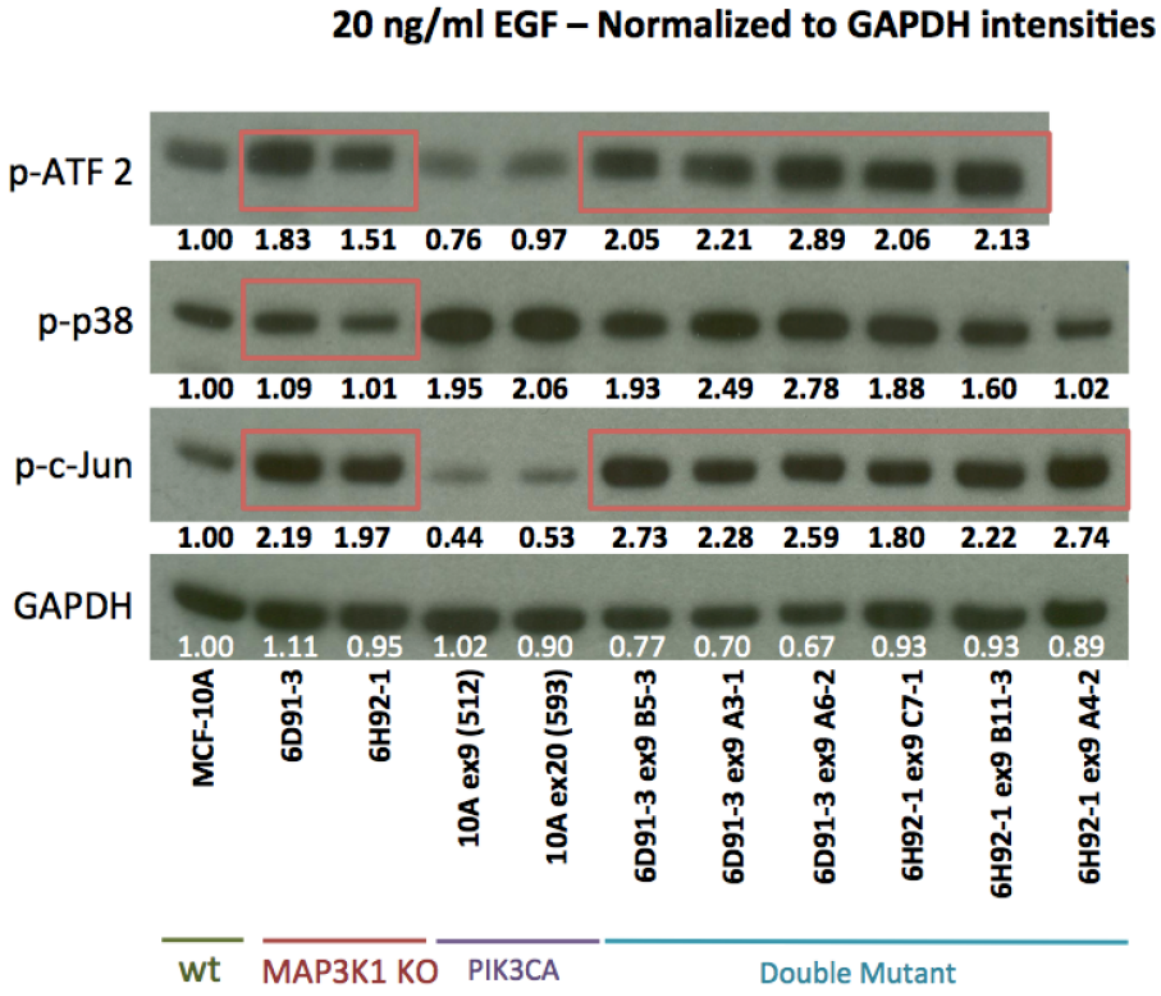


Figure 14. Downstream MAP3K1 signaling in either wild-type MCF-10A parental cells (left, green), MAP3K1 knockout cells (red), PIK3CA activated cells (purple), or double mutant cell lines in which both MAP3K1 has been knocked out and PIK3CA activated (right, blue). ***MAP3K1*** loss in MCF-10A cells appears to be associated with increased ATF-2 (activating transcription factor 2) activation, decreased p38 (a mitogen-activated protein kinase) activation, and increased c-Jun activation



REFERENCES

Abukhdeir, A. M., B. G. Blair, K. Brenner, B. Karakas, H. Konishi, J. Lim, V. Sahasranaman, Y. Huang, J. Keen, N. Davidson, M. I. Vitolo, K. E. Bachman and B. H. Park (2006). "Physiologic estrogen receptor alpha signaling in non-tumorigenic human mammary epithelial cells." Breast Cancer Res Treat **99**(1): 23-33.

Banerji, S., K. Cibulskis, C. Rangel-Escareno, K. K. Brown, S. L. Carter, A. M. Frederick, M. S. Lawrence, A. Y. Sivachenko, C. Sougnez, L. Zou, M. L. Cortes, J. C. Fernandez-Lopez, S. Peng, K. G. Ardlie, D. Auclair, V. Bautista-Pina, F. Duke, J. Francis, J. Jung, A. Maffuz-Aziz, R. C. Onofrio, M. Parkin, N. H. Pho, V. Quintanar-Jurado, A. H. Ramos, R. Rebollar-Vega, S. Rodriguez-Cuevas, S. L. Romero-Cordoba, S. E. Schumacher, N. Stransky, K. M. Thompson, L. Uribe-Figueroa, J. Baselga, R. Beroukhim, K. Polyak, D. C. Sgroi, A. L. Richardson, G. Jimenez-Sanchez, E. S. Lander, S. B. Gabriel, L. A. Garraway, T. R. Golub, J. Melendez-Zajgla, A. Toker, G. Getz, A. Hidalgo-Miranda and M. Meyerson (2012). "Sequence analysis of mutations and translocations across breast cancer subtypes." Nature **486**(7403): 405-409.

Cancer Genome Atlas, N. (2012). "Comprehensive molecular portraits of human breast tumours." Nature **490**(7418): 61-70.

Ciriello, G., R. Sinha, K. A. Hoadley, A. S. Jacobsen, B. Reva, C. M. Perou, C. Sander and N. Schultz (2013). "The molecular diversity of Luminal A breast tumors." Breast Cancer Res Treat **141**(3): 409-420.

Cowell, J. K., J. LaDuca, M. R. Rossi, T. Burkhardt, N. J. Nowak and S. Matsui (2005). "Molecular characterization of the t(3;9) associated with immortalization in the MCF10A cell line." Cancer Genet Cytogenet **163**(1): 23-29.

Easton, D. F., K. A. Pooley, A. M. Dunning, P. D. Pharoah, D. Thompson, D. G. Ballinger, J. P. Struewing, J. Morrison, H. Field, R. Luben, N. Wareham, S. Ahmed, C. S. Healey, R. Bowman, S. collaborators, K. B. Meyer, C. A. Haiman, L. K. Kolonel, B. E. Henderson, L. Le Marchand, P. Brennan, S. Sangrajrang, V. Gaborieau, F. Odefrey, C. Y. Shen, P. E. Wu, H. C. Wang, D. Eccles, D. G. Evans, J. Peto, O. Fletcher, N. Johnson, S. Seal, M. R. Stratton, N. Rahman, G. Chenevix-Trench, S. E. Bojesen, B. G. Nordestgaard, C. K. Axelsson, M. Garcia-Closas, L. Brinton, S. Chanock, J. Lissowska, B. Peplonska, H. Nevanlinna, R. Fagerholm, H. Eerola, D. Kang, K. Y. Yoo, D. Y. Noh, S. H. Ahn, D. J. Hunter, S. E. Hankinson, D. G. Cox, P. Hall, S. Wedren, J. Liu, Y. L. Low, N. Bogdanova, P. Schurmann, T. Dork, R. A. Tollenaar, C. E. Jacobi, P. Devilee, J. G. Klijn, A. J. Sigurdson, M. M. Doody, B. H. Alexander, J. Zhang, A. Cox, I. W. Brock, G. MacPherson, M. W. Reed, F. J. Couch, E. L. Goode, J. E. Olson, H. Meijers-Heijboer, A. van den Ouweland, A. Uitterlinden, F. Rivadeneira, R. L. Milne, G. Ribas, A. Gonzalez-Neira, J. Benitez, J. L. Hopper, M. McCredie, M. Southey, G. G. Giles, C. Schroen, C. Justenhoven, H. Brauch, U. Hamann, Y. D. Ko, A. B. Spurdle, J. Beesley, X. Chen, kConFab, A. M. Group, A. Mannermaa, V. M. Kosma, V. Kataja, J. Hartikainen, N. E. Day, D. R. Cox and B. A. Ponder (2007). "Genome-wide association study identifies novel breast cancer susceptibility loci." Nature **447**(7148): 1087-1093.

Ellis, M. J., L. Ding, D. Shen, J. Luo, V. J. Suman, J. W. Wallis, B. A. Van Tine, J. Hoog, R. J. Goiffon, T. C. Goldstein, S. Ng, L. Lin, R. Crowder, J. Snider, K. Ballman, J. Weber, K. Chen, D. C. Koboldt, C. Kandoth, W. S. Schierding, J. F. McMichael, C. A. Miller, C. Lu, C. C. Harris, M. D. McLellan, M. C. Wendl, K. DeSchryver, D. C. Allred, L. Esserman, G. Unzeitig, J. Margenthaler, G. V. Babiera, P. K. Marcom, J. M. Guenther, M. Leitch, K. Hunt, J. Olson, Y. Tao, C. A. Maher, L. L. Fulton, R. S. Fulton, M. Harrison, B. Oberkfell, F. Du, R. Demeter, T. L. Vickery, A. Elhammali, H. Piwnica-Worms, S. McDonald, M. Watson, D. J. Dooling, D. Ota, L. W. Chang, R. Bose, T. J. Ley, D. Piwnica-Worms, J. M. Stuart, R. K. Wilson and E. R. Mardis (2012). "Whole-genome analysis informs breast cancer response to aromatase inhibition." Nature **486**(7403): 353-360.

Fuchs, S. Y., V. Adler, M. R. Pincus and Z. Ronai (1998). "MEKK1/JNK signaling stabilizes and activates p53." Proc Natl Acad Sci U S A **95**(18): 10541-10546.

Gustin, J. P., B. Karakas, M. B. Weiss, A. M. Abukhdeir, J. Lauring, J. P. Garay, D. Cosgrove, A. Tamaki, H. Konishi, Y. Konishi, M. Mohseni, G. Wang, D. M. Rosen, S. R. Denmeade, M. J. Higgins, M. I. Vitolo, K. E. Bachman and B. H. Park (2009). "Knockin of mutant PIK3CA activates multiple oncogenic pathways." Proc Natl Acad Sci U S A **106**(8): 2835-2840.

Hsu, P.D., Scott, D.A., Weinstein, J.A., Ran, F.A., Konermann, S., Agarwala, V., Li, Y., Fine, E.J., Wu, X., Shalem, O., Cradick, T.J., Marraffini, L.A., Bao, G., Zhang, F (2013). "DNA targeting specificity of RNA-guided Cas9 nucleases." Nat Biotechnol **31**(9): 827-832.

Hu, P., Q. Huang, Z. Li, X. Wu, Q. Ouyang, J. Chen and Y. Cao (2014). "Silencing MAP3K1 expression through RNA interference enhances paclitaxel-induced cell cycle arrest in human breast cancer cells." Mol Biol Rep **41**(1): 19-24.

Inic, Z., M. Zegarac, M. Inic, I. Markovic, Z. Kozomara, I. Djurisic, I. Inic, G. Pupic and S. Jancic (2014). "Difference between Luminal A and Luminal B Subtypes According to Ki-67, Tumor Size, and Progesterone Receptor Negativity Providing Prognostic Information." Clin Med Insights Oncol **8**: 107-111.

Jin, C., J. Chen, Q. Meng, V. Carreira, N. N. Tam, E. Geh, S. Karyala, S. M. Ho, X. Zhou, M. Medvedovic and Y. Xia (2013). "Deciphering gene expression program of MAP3K1 in mouse eyelid morphogenesis." Dev Biol **374**(1): 96-107.

Johnson, K.R., Young, K.K., Fan, W (1999). "Antagonistic interplay between antimitotic and G1-S arresting agents observed in experimental combination therapy." Clin Cancer Res **5**(9): 2559-2565.

Laprise P., Langlois M.J., Boucher M.J., Jobin C., and Rivard N (2004). " Down-regulation of MEK/ERK signaling by E-cadherin-dependent PI3K/Akt pathway in differentiating intestinal epithelial cells." J Cell Physiol **1**:32-9.

Pham, T. T., S. P. Angus and G. L. Johnson (2013). "MAP3K1: Genomic Alterations in Cancer and Function in Promoting Cell Survival or Apoptosis." Genes Cancer **4**(11-12): 419-426.

Riaz, M., F. Elstrodt, A. Hollestelle, A. Dehghan, J. G. Klijn and M. Schutte (2009). "Low-risk susceptibility alleles in 40 human breast cancer cell lines." BMC Cancer **9**: 236.

Shearin AL, Hedan B, Cadieu E, Erich SA, Schmidt EV, Faden DL, Cullen J, Abadie J, Kwon EM, Gröne A, Devauchelle P, Rimbault M, Karyadi DM, Lynch M, Galibert F, Breen M, Rutteman GR, André C, Parker HG, and Ostrander EA (2012). "The MTAP-CDKN2A locus confers susceptibility to a naturally occurring canine cancer." Cancer Epidemiol Biomarkers Prev. **7**:1019-27

Siegel R., Miller K.D., Jemal A (2015). "Cancer Statistics, 2015" CA Cancer J Clin **1**: 5-29.

Stephens, P. J., P. S. Tarpey, H. Davies, P. Van Loo, C. Greenman, D. C. Wedge, S. Nik-Zainal, S. Martin, I. Varela, G. R. Bignell, L. R. Yates, E. Papaemmanuil, D. Beare, A. Butler, A. Cheverton, J. Gamble, J. Hinton, M. Jia, A. Jayakumar, D. Jones, C. Latimer, K. W. Lau, S. McLaren, D. J. McBride, A. Menzies, L. Mudie, K. Raine, R. Rad, M. S. Chapman, J. Teague, D. Easton, A. Langerod, C. Oslo Breast Cancer, M. T. Lee, C. Y. Shen, B. T. Tee, B. W. Huimin, A. Broeks, A. C. Vargas, G. Turashvili, J. Martens, A. Fatima, P. Miron, S. F. Chin, G. Thomas, S. Boyault, O. Mariani, S. R. Lakhani, M. van de Vijver, L. van 't Veer, J. Foekens, C. Desmedt, C. Sotiriou, A. Tutt, C. Caldas, J. S. Reis-Filho, S. A. Aparicio, A. V. Salomon, A. L. Borresen-Dale, A. L. Richardson, P. J. Campbell, P. A. Futreal and M. R. Stratton (2012). "The landscape of cancer genes and mutational processes in breast cancer." Nature **486**(7403): 400-404.

Yan, X. S., J. Barnholtz-Sloan, X. Chu, L. Li, R. Colonie, J. Webster, D. Smelser, N. Patel, J. Prichard and A. Stark (2013). "Adiposity, inflammation, genetic variants and risk of post-menopausal breast cancer findings from a prospective-specimen-collection, retrospective-blinded-evaluation (PRoBE) design approach." Springerplus **2**: 638.

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CORE EXPERTISE/SUMMARY

- Over ten years of experience in the cancer genetics/genomics field in human studies
- 11 peer-reviewed papers including two as first author and one review; 1 review in progress
- Extensive experience in molecular biology procedures and technologies including all steps of dideoxy sequencing, cloning methods, expression assays, tissue culture, and cell maintenance
- First researcher to create a knock-out cell line for the breast-cancer-relevant gene MAP3K1 in both the normal breast cell line MCF-10A and the breast cancer cell line MCF-7 through the use of recombinant adeno-associated virus genome editing as well as CRISPR
- Advanced capabilities in epidemiological practices including study design, carrying out studies, data collection, and statistical analysis using a number of software packages
- Highly proficient in biomedical research literature searching and knowledge summation

EDUCATION

- M.S., Human Genetics, The Johns Hopkins University School of Medicine Program in Human Genetics, 2016 (***in process***)
- M.P.H., Epidemiology, The University of Michigan School of Public Health, 2011
- B.A., History, The University of Michigan College of Literature, Science, and Arts, 2008

RESEARCH EXPERIENCE

National Cancer Institute Fellow
The National Institutes of Health
2015-present

- Investigating the role of a newly developed antibody in the treatment of breast cancer including the optimal dosage, combination with other therapies, and profile of who would benefit most. Includes looking at the how the antibody works on a mechanistic level and genetic/proteomic factors leading to sensitivity or resistance
- Investigating the genetic and proteomic contributors to TNF-related apoptosis-inducing ligand (TRAIL) resistance in triple negative breast cancer.

Graduate Research Scientist
Johns Hopkins University School of Medicine Human Genetics Graduate Program (2 labs)

2014-2015

- Managed a project investigating the rare human cancer histiocytic sarcoma using the Bernese Mountain Dog as a model
- Analyzed next-generation sequence data and genome wide association data to narrow down the region of interest to a 5-Mb region. Further refined the region through investigative sequencing of cases and controls to a handful of SNPs that are potentially causative for the cancer
- Initiated a project exploring why two similar breeds of dog, the Bernese Mountain Dog and the Greater Swiss Mountain Dog, develop cancer at highly different rates
- Wrote two review papers; one on the genomics of dog domestication and the other on the utility of the domestic dog as a model for human cancers in process

2011-2014

- Managed a project looking into the effects of ***MAP3K1*** disruption in breast cancer, a gene which had commonly been seen as mutated in women with luminal subtypes
- Created a number of ***MAP3K1***-null cell lines in order to investigate effects of the gene on the pathway level, individual protein levels, cellular function, morphology, chemotherapeutic resistance, and other classic cancer phenotypes
- Discovered that while there were no haploinsufficient effects of losing one allele, losing both alleles resulted in aberrant morphology as well as differential growth rates and chemotherapeutic susceptibility
- Followed up my investigation with mutations of other genes in the map kinase pathway in other cell lines
- Standardized a number of protocols pertaining to the assays performed in this newly started lab
- Helped train undergraduate and summer students
- Teacher's assistant for graduate course entitles "Introduction to Genetics"

Masters Research Scientist
The University of Michigan School of Public Health Epidemiology Program
2009-2011

- Managed a project exploring the effect of the androgen receptor polyglutamine tract on the development of male breast cancer in North African populations
- Designed study from the beginning including inclusion and exclusion criteria of cases and controls, established contacts in Egyptian and Moroccan hospitals where male breast cancer is especially common, and traveled to Egypt to procure viable control tissue
- Developed and standardized a technique using amplified fragment length polymorphisms labeled with a fluorescent dye to precisely determine gene and receptor length in study subjects
- Discovered that longer androgen receptors are associated with the development of male breast cancer
- Published two first-author papers about breast cancer as well as assisted on various other projects that resulted in secondary authorships
- Involved in recruiting and training students while in the cancer epidemiology education in special populations program (CEESP)

Undergraduate Research Technician
The University of Michigan, Department of Human Genetics
2006-2008

- Genotyped and tended to transgenic mice colonies to determine androgen receptor length categories to determine association with female breast cancer
- General laboratory maintenance

Student Researcher
The William Beaumont Hospital Department of Oncology, Royal Oak, MI
2005-2007

- Gathered information on breast cancer patients to input into databases
- Created databases to organize to multiple relevant data points for each patient/project
- Helped physicians write and prepare abstracts, manuscripts, and lectures
- Assisted and observed radiation oncologists in all aspects of their job
- Organized office procedures to ensure an efficient work environment
- Co-authorships on 4 papers

TEACHING EXPERIENCE

Genetics Instructor
Johns Hopkins Center for Talented Youth
2015

- Taught gifted high school students the subject of genetics in a classroom setting
- Designed lesson plans, classroom activities, quizzes/tests, and laboratory projects
- Monitored student progress via assessment methods and one-on-one interaction and tailored lessons to ensure each student obtained the appropriate information
- Supervised laboratory activities, instructed students on laboratory procedure and experimental technique, and helped students understand the processes involved
- Worked closely with a teacher's assistant to ensure proper classroom management
- Partook in parent-teacher conferences to provide feedback to students and their families regarding their progress, constructive criticism, and important takeaways for proceeding in the science field

AWARDS

- Awarded second place in the poster competition among graduate students at The Maryland Genetics, Epidemiology, and Medicine Training Program Genetics Research Day

TECHNICAL SKILLS SUMMARY

Molecular Biology
Gene cloning and vector design, gene expression assays (Western blotting, RT-PCT), PCR, qPCR, Sanger sequencing with the 3730xl DNA Analyzer
Cell Culture
Mammalian cell culture using cancer and normal cell lines, growth assays, drug sensitivity assays, morphology assays, gene targeting
Computer Skills
Proficient in SAS, R, ImageJ, Haploview, Plink

PROFESSIONAL SOCIETIES

2014-present	Genetics Society of America
2016-present	American Association of Cancer Researchers

INVITED PRESENTATIONS AND ABSTRACTS

- Invited to talk and present at the International Cancer Education Conference in Cairo, Egypt; October 2010
- Invited to talk and present at the American Association for Cancer Educators conference in Buffalo, NY; September, 2011

PUBLICATIONS

1. Vicini FA, Antonucci JV, Goldstein N, Wallace M, Kestin L, Krauss D, Kunzmann J, **Gilbert S**, Schell S. The use of molecular assays to establish definitively the clonality of ipsilateral breast tumor recurrences and patterns of in-breast failure in patients with early-stage breast cancer treated with breast- conserving therapy. ***Cancer***. 2007 Apr 1;109(7):1264-72. PMID: 17372920.

2. Vicini FA, Antonucci JV, Wallace M, **Gilbert S**, Goldstein NS, Kestin L, Chen P, Kunzman J, Boike T, Benitez P, Martinez A. Long-term efficacy and patterns of failure after accelerated partial breast irradiation: a molecular assay-based clonality evaluation. ***Int J Radiat Oncol Biol Phys***. 2007 Jun 1;68(2):341-6. PMID: 17306933.

3. Chao KK, Vicini FA, Wallace M, Mitchell C, Chen P, Ghilezan M, **Gilbert S**, Kunzman J, Benitez P, Martinez A. Analysis of treatment efficacy, cosmesis, and toxicity using the MammoSite breast brachytherapy catheter to deliver accelerated partial-breast irradiation: the william beaumont hospital experience. ***Int J Radiat Oncol Biol Phys***. 2007 Sep 1;69(1):32-40. PMID: 17467920.

4. Vicini FA, Chen P, Wallace M, Mitchell C, Hasan Y, Grills I, Kestin L, Schell S, Goldstein NS, Kunzman J, **Gilbert S**, Martinez A. Interim cosmetic results and toxicity using 3D conformal external beam radiotherapy to deliver accelerated partial breast irradiation in patients with early-stage breast cancer treated with breast-conserving therapy. ***Int J Radiat Oncol Biol Phys***. 2007 Nov 15;69(4):1124- 30. PMID: 17967306.

5. **Gilbert SF**, Soliman AS, Iniesta M, Eissa M, Hablas A, Seifeldin IA, Strahley A, Banerjee M, Merajver SD. Androgen receptor polyglutamine tract length in Egyptian male breast cancer patients. ***Breast Cancer Res Treat***. 2011 Sep;129(2):575-81. PMID: 21505847.

6. **Gilbert SF**, Soliman AS, Karkouri M, Quinlan-Davidson M, Strahley A, Eissa M, Dey S, Hablas A, Seifeldin IA, Ramadan M, Benjaafar N, Toy K, Merajver SD. Clinical profile, BRCA2 expression, and the androgen receptor CAG repeat region in Egyptian and Moroccan male breast cancer patients. ***Breast Dis***. 2011;33(1):17-26. PMID: 22142662.

7. Veruttipong D, Soliman AS, **Gilbert SF**, Blachley TS, Hablas A, Ramadan M, Rozek LS, Seifeldin IA. Age distribution, polyps and rectal cancer in the Egyptian population-based cancer registry. ***World J Gastroenterol***. 2012 Aug 14;18(30):3997-4003. PMID: 22912550.

8. Hossain MZ, **Gilbert SF**, Patel K, Ghosh S, Bhunia AK, Kern SE. Biological clues to potent DNA- damaging activities in food and flavoring. ***Food Chem Toxicol***. 2013 May;55:557-67. PMID: 23402862.

9. Beaver JA, Gustin JP, Yi KH, Rajpurohit A, Thomas M, **Gilbert SF**, Rosen DM, Park BH, Lauring J. ***PIK3CA*** and ***AKT1*** mutations have distinct effects on sensitivity to targeted pathway inhibitors in an isogenic luminal breast cancer model system. ***Clin Cancer Res***. 2013 Oct 1;19(19):5413-22. PMID: 23888070.

10. Ghosh S, Bhunia AK, Paun BC, **Gilbert SF**, Dhru U, Patel K, Kern, SE. Genome annotation by shotgun inactivation of a native gene in

hemizygous cells: application to ***BRCA2*** with implication of hypomorphic variants. ***Hum Mutat.*** 2015 Feb;36(2):260-9. PMID: 25451944.

11. Parker HG, **Gilbert SF.** From caveman companion to medical innovator: genomic insights into the origin and evolution of domestic dogs. ***Adv Genomics Genet.*** 2015 Feb 28;5:239-255. doi:10.2147/AGG.S57678.